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ATTY.'S DOCKET: KORSGREN=1

In re Application of:	)	Art Unit: 1614
Olle KORSGREN et al	)	Examiner: Donna JAGOE
Appln. No.: 09/890,936	)	Washington, D.C.
Date Filed: November 7, 2001	)	Confirmation No. 9165
For: NOVEL USE WITHIN TRANSPLANTATION SURGERY	)	

SECOND DECLARATION UNDER 37 CFR 1.132

Olle Korsgren, Bo Nilsson and Rolf Larsson hereby each solemnly declare as follows:

We are co-applicants and co-inventors of the above-identified application. We each have both M.D. and Ph.D. degrees. We earlier submitted a declaration in this application, also under 37 CFR 1.132, which we reiterate and confirm, and which has attached thereto our CV's which are made a part of this declaration as well.

Each of us confirms familiarity with the prior art documents cited and relied upon by the U.S. patent examiner and the commentary of the U.S. examiner in conjunction with reliance on such prior art citations. We amplify our earlier declaration as follows:

Encapsulation (also referred to as microencapsulation) implies that the islets are confined

within a polymer membrane that is <u>not in direct contact</u> with the islets. Extensive efforts have been spent on preparing microcapsules primarily using alginates as the capsules-forming material in accordance with what is disclosed in Wagner and Soon-Shiong.

A recent article (ref. 1), Dufrane et al[1], is representative for the current state-of-the-art. As illustrated in Fig 1 in the cited article, an optimal capsule should be a sphere with a diameter of 650- 700 µm. Wagner specifies a diameter of 500 µm (p. 6, item 2, l.3-4 in translated document).

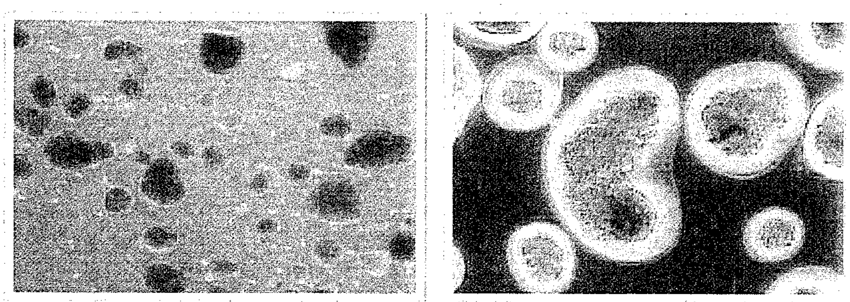
Considering that the islets have a size of 50-300 µm, we state as fact beyond any doubt that one or several islets will be enclosed in each microcapsule with considerable dead space between the islet(s) and the enclosing membrane.

Even with the most advanced technology using polyethylene glycol as the capsule-forming material, as being used by e.g. Novocell Inc. (see www.novocell.com), the capsules of the prior art leave a dead space of 25-50 µm between the cell surface and the polymer membrane (see fig. 1 below). The dead space creates delayed response times due to the fact that glucose must first diffuse through the membrane,

[1] 1. Dufrane D., Goebbels R-M., Saliez A., Guiot Y., and Gianello P.: Six-months survival of microencapsulated pig islets and alginate biocompatibility in primates. Proof-of-concept. Transplantation, 9, 1345-1353, 2006

and then the glucose has to be transported by a concentration gradient across the dead space until it reaches the cell surface.

Fig 1. (microphotographs obtained at www.novocell.com)



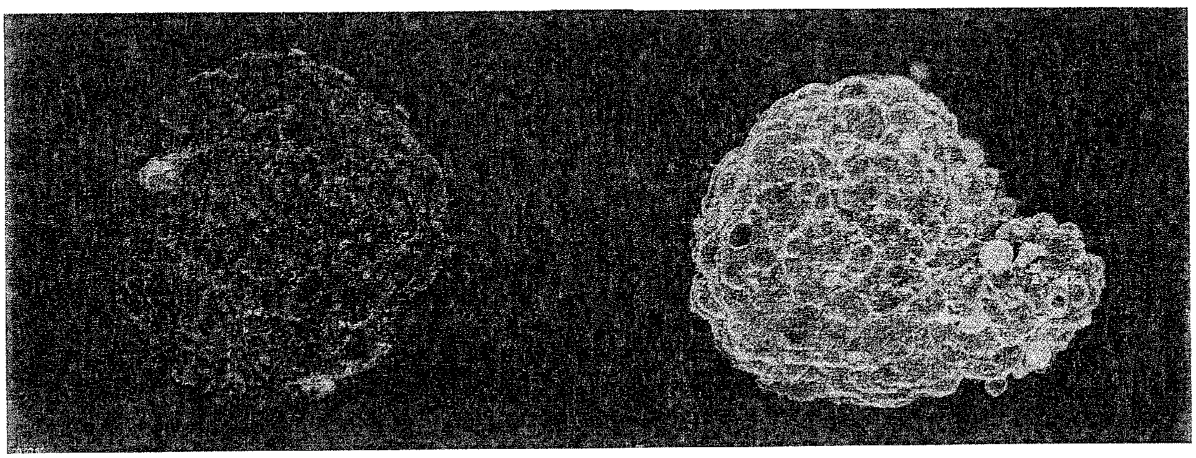
Purified Human Islets **Encapsulated Islets with PEG Conformal Coatings**

The insulin response by the cell will be delayed and the occurrence of insulin in the blood is further delayed by the dead space and diffusion through the membrane. The end result is that the actual insulin response in blood will be too late and may even induce hypoglycemia, if increased levels of insulin are generated too late when the glucose levels are already going down.

On the contrary, in our invention, the material used, e.g. Corline Heparin Conjugate, is adsorbed directly in close contact with individual islets with no dead space, whereby glucose in blood interacts directly with the islet cells so that a physiological response with regard to release of insulin is achieved. The following picture (Fig. 2), prepared in our laboratory, shows one islet cell coated with

heparin according to our invention and examined by confocal microscopy using fluorescently labelled antithrombin that binds to heparin. It is evident that the coating follows the contour of the cell and is in direct contact with the cell surface.

Fig. 2



Islet of Langerhans, non-modified (left) and coated with the Corline Heparin Surface (right). Antithrombin (which has strong affinity for heparin) labelled with a fluorescent dye, Alexa 488, was allowed to adsorb to the surface. Islets were then examined in a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany) equipped with an Axiovert 200 microscope stand. Z-stacks of the islet surfaces were acquired using the 488nm laser line, a 20X objective and a 505-550 BP filter. Three-dimensional projections of the acquired z-stacks were analyzed using Imaris software (Bitplane, Zurich, Switzerland). The non-modified islet (left) only displays weak autofluorescens, whereas the adsorbed heparin is

visualised as a smooth and coherent coating that follows the contours of the islet.

There is another distinct difference between encapsulated islets such as those of Wagner and Soon-Shiong, and heparin coated islets according to our invention that needs to be emphasised. The spherical membrane that encapsulates the islets of the prior art is an insoluble polymer with a cross-longitudinal network of bonds (thus it forms a membrane barrier between the islet surface and the surroundings), whereas the material we use, e.g. Corline Heparin Conjugate, is a soluble macromolecule that is adsorbed as discrete molecules onto the cell surface with no cross-longitudinal linkages being formed between the conjugate molecules (thus no membrane barrier is formed between the islet surface and the surroundings).

Soon-Shiong et al discusses "microcapsules prepared from crosslinkable polysaccharides, polycations and/or lipids and use therefor" (title of the patent). No part of the Soon-Shiong document demonstrates or discusses the use of heparin or heparin conjugates for coating islets without polymerization (or crosslinking, alternatively gel formation). On the contrary, examples 1-7, 14-17, 19, 21-25, 27-28 and 30-32 are specifically related to crosslinking, gels, polymerization etc. Therefore, Soon-Shiong et al do something

quite different from our invention, and this document leads away from our invention.

In their patent application, Wagner et al mention islets of Langerhans in Claim 3 and heparin used to antagonize agglomeration in claim 7. However, no disclosure of our present invention can be found in the patent application of Wagner et al. On the contrary, Wagner et al only describe the use of macro- and micro-encapsulation of islet cells, and thereby leads or directs worker in our field away from our invention.

As clearly explained in our previous Declaration, the encapsulating membrane composed of an insoluble polymer proposed by Wagner et al and Soon-Shiong et al constitutes a barrier to immunologically active cells and molecules, whereas no such barrier is created by adsorption of the heparin, e.g. Corline Heparin Conjugate. We reiterate with strong emphasis that it is absolutely clear and certain that the chemical constitution and diffusion characteristics are fundamentally different between the two methods, and that there is no chemical or technical rationale on basis of which one could accurately maintain that adsorption of the Corline Heparin Conjugate would lead to encapsulation in the sense of Wagner et al and Soon-Shiong et al.

We hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Six-Month Survival of Microencapsulated Pig Islets and Alginate Biocompatibility in Primates: Proof of Concept

Denis Dufrane,[1] Rose-Marie Goebbels,[1] Alain Saliez,[1] Yves Guiot,[2] and Pierre Gianello[1,3]

Background. Pig islets xenotransplantation remains associated with a strong humoral and cellular xenogeneic immune responses. The aim of this study was to assess the long-term biocompatibility of alginate encapsulated pig islets after transplantation in primates

Methods. Adult pig islets encapsulated in alginate under optimal conditions (n=7) or not (n=5) were transplanted under the kidney capsule of nondiabetic Cynomolgus maccacus. Additional primates received empty capsules (n=1) and nonencapsulated pig islets (n=2) as controls. Capsule integrity, cellular overgrowth, pig islet survival, porcine C-peptide and anti-pig IgM/IgG antibodies were examined up to 6 months after implantation

Results. Nonencapsulated islets and islets encapsulated in nonoptimal capsules were rapidly destroyed. In seven primates receiving perfectly encapsulated pig islets, part of the islets survived up to 6 months after implantation without immunosuppression. Porcine C-peptide was detected after 1 month in 71% of the animals. The majority of grafts (86%) were intact and completely free of cellular overgrowth or capsule fibrosis. Explanted capsules, after 135 (n=2/2) and 180 (n=2/3) days, demonstrated residual insulin content and responses to glucose challenge (stimulation index of 2.2). Partial islet survival was obtained despite an elicited anti-pig IgG humoral response

Conclusions. Optimal alginate encapsulation significantly prolonged adult pig islet survival into primates for up to 6 months, even in the presence of antibody response.

Keywords: Pig islet transplantation, Xenotransplantation, Microencapsulation, Primate, Biocompatibility.

(*Transplantation* 2006;81: 1345–1353)

The need for permanent immunosuppression and the shortage of pancreas donors remain major hurdles to the widespread use of pancreatic islet cell transplantation to curing type I diabetes (*1, 2*).

In order to protect islet cells from immune reaction without the need for chronic immunosuppression, microencapsulation has been considered and demonstrated to be effective in mice (*3–6*). In most studies, islets have been encased in alginate-polylysine-alginate microcapsules (*7*) but the cytotoxicity of polyaminoacid and the mechanical instability of microcapsules limit their application (*5, 8, 9*). Several groups have recently reported that encapsulation in a simple microbead made of alginate is able to protect pig pancreatic cells against allo- or xenorejection in diabetic mice (*4, 5, 10, 11*). However, a question remains about the putative application of this method in a preclinical large animal model.

In order to overcome the shortage of human pancreases, pig islets represent an important therapeutic alternative (*12*). Although the strong humoral and cellular xenogeneic immune responses appear difficult to overcome (*13*), pig islet survival has been demonstrated for up to 53 days in primates by using anti-thymocyte globulins (*14*). Recent results reported by B. Hering (personal communication) seem, however, to show that the use of a heavy immunosuppressive regimen (5 or 6 drugs), allows pig islets to survive in primates for up to months. Such an immunosuppressive regimen seems unlikely to be acceptable in humans, but these results are, however, encouraging. This result suggests that other alternatives such as microencapsulation should be evaluated in the pig to primate model.

This study is the first to assess the factors involved in successful pig islet immunoprotection by alginate capsule when transplanted in non-diabetic primates without any immunosuppression. The impact of several parameters (stabilization, culture period) involved in in vivo and in vitro alginate capsule biocompatibility, as well as the ability of alginate capsules to protect pig islets in primates from the strong xe-

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nogeneic immune response were evaluated for up to six months.

MATERIALS AND METHODS

Pig Islet Cell Isolation and Encapsulation

Adult pig pancreases were harvested from Landrace pigs (>200 kg, n=10) at the local slaughterhouse (Centre A. de Marbaix, Louvain-la-Neuve, Mr. Collignon) and islets were isolated using a previously described technique (*15*).

Freshly isolated pig islets were encapsulated in an SLM 100 alginate matrix (Batch 110064, FMC BioPolymer, Drammen, Norway) containing a high concentration of mannuronic acid (High-M, 56%). Freeze-dried alginate (viscosity: 174 mPa.s; endotoxin <25 EU/gram), was diluted in a MOPS 1X washing buffer (Inotech Encapsulation AG, Dottikon, Switzerland) at a concentration of 1% w/v. Pig islet cells were suspended in alginate at a concentration of 10,000 islet cells/ml and encapsulation was performed by the Inotech Encapsulation AG device (serial number: LS-01.005; Dottikon, Switzerland) (Dufrane et al., manuscript submitted). Quality of capsule was microscopically evaluated (on 100 capsules samples) in order to determine the capsule diameter and the percentage of non-well shaped and broken capsules.

Microcapsule Culture Condition

In order to obtain optimal stability of microcapsule, several $CaCl_2$ concentrations were tested in culture. Empty capsules were cultured in HAM-F10 medium (NV Invitrogen, Merelbeke, Belgium) and supplemented with either 0.3, 1.8, 2.5 or 5 mM $CaCl_2$ for 2, 18 and 24 hr of incubation. One hundred capsule diameters, for each series, were examined on an inverted phase contrast microscope with a calibrated grid in the eyepiece.

Animal Recipients

Non-diabetic Cynomolgus monkeys (3–6 years old; 4–7 kg, n=15) were used as recipients and were housed according to the guidelines of the Belgian Ministry of Agriculture and Animal Care. All procedures were approved by the local Ethics Committee for Animal Care of the Université Catholique de Louvain. Three experimental groups were designed: one primate received empty capsules (Negative Control, Ctrl−); 2 primates were transplanted with non-encapsulated pig islets (Positive Control, Ctrl+) and 12 primates were transplanted with pig encapsulated islets (Experimental Group).

In this latter group, two primates received pig encapsulated islets which had not been cultivated for 18 hr prior to transplantation (without capsule stabilization), three animals received pig encapsulated islets which had been cultivated for 18 hr prior to transplantation but in medium supplemented by 10% v/v of FBS (NV Invitrogen, Merelbeke, Belgium), and finally, seven primates received pig encapsulated islets after an 18 hr period of culture in a medium free of serum.

Transplantation of Microencapsulated Islets

Kidney subcapsular space was chosen since it seems a less immunoreactive implantation site for encapsulated xenoislets than peritoneum (Dufrane et al., September 2005, submitted in Biomaterials).

After anesthesia, kidneys were exposed, by a midline laparotomy, and a 20-gauge catheter (Becton Dickinson, Aalst, Belgium) was placed between the kidney parenchyma and the capsule. Each animal received 15,000 microencapsulated islets equivalent to (IEQ)/kg of body recipient, collected in a 10 ml syringe. Positive control animals received the same number of non-encapsulated pig islets under the kidney capsule. Negative control animal received a volume of empty capsules corresponding to the volume of 15,000 IEQ/kg encapsulated pig islets (a mean of 7 ml). Each graft was transplanted under the capsule of one kidney per primate.

Animal Follow-up

Blood glucose was monitored during the first 24 hr posttransplantation to avoid hypoglycaemia. Blood samples were taken (via the femoral vein) prior to transplantation (three times to determine the Porcine C-peptide baseline) and 1 hr, days 1, 7, 30, 60, 90, 135 and 180 posttransplantation. Porcine C-peptide (Linco Research, Nuclilab BV, BB EDE, The Netherlands), anti-porcine antibodies (see below) and creatinine (Kodak Ektachem DTSC II; Ortho-Clinical Diagnostics, INC, Rochester, NY, USA) levels were measured on primate sera. Body Weight was monitored weekly.

Detection of Anti-porcine IgM and IgG

Porcine lymphocytes were used as antigenic targets for the detection of anti-porcine antibodies. Ten millilitres of heparinized blood, obtained from Belgium landrace pigs (12–15 weeks old), were diluted in 25 ml of RPMI and lymphocytes were recovered after centrifugation across a density gradient on lymphocyte separation medium (LSM; Biochrom AG, Berlin, Germany). Specific anti-porcine IgM or IgG antibodies were assessed by Flow Cytometry using a fluorescence-activated cell sorter (Facsort, Becton Dickinson, BD Erembodegem, Aalst, Belgium). The 8×10^5 porcine lymphocytes/tube were incubated 30 min with 20 μl of serum from each experimental primate (primate serum was used without dilution and decomplemented at 56°C). After incubation, at 4°C, three washes were performed with HBSS solution. FITC-labeled rat anti-human IgM (LO-BM2) and IgG (LO-HG22) antibodies (IMEX, Université Catholique de Louvain, Brussels, Belgium) were incubated with lymphocytes and washed three times after 30 min of incubation. These data were acquired and processed using CELL Quest software (BD Bioscience). The mean fluorescence intensity (MFI) and percentage of lymphocyte binding anti-porcine antibody were assessed.

Assessment of Explanted Capsules

Under anesthesia, nephrectomy was performed and the microcapsules were recovered 30, 90, 135 and 180 days after transplantation. Macroscopic quality of the graft was assessed as the following score in 3 groups: no graft fibrosis (0%); >50% of entrapped capsules in fibrosis and total graft fibrosis (100%). The proportion of capsule overgrowth, which was defined as the percentage of capsules with more than 50% of surface covered by cells, was microscopically evaluated on a mean of 400 capsules/explanted graft. The proportion of broken capsules and the proportion of capsules containing dithyzone (DTZ) staining islets were also evaluated microscopically.

The ability of encapsulated adult pig islets to produce insulin 135 and 180 days after transplantation was assessed by a static glucose challenge. Prior to the test, explanted capsules had been cultivated in 75 cm^2 flasks, for 15 hr, on RPMI 1640 glucose 5 mM supplemented with 10% FBS at 37°C. After culture, encapsulated pig islets were removed from the flasks, washed three times with RPMI 1640 glucose 5 mM and incubated for 24 hr, in RPMI 1640 supplemented with glucose 5 mM, 15 mM or 15 mM + Forskolin 1 μM (37°C). After incubation, supernatants were collected and stored at −20°C until measurement of insulin by a double antibody radioimmunoassay. Insulin content of the microencapsulated pig islets was measured in samples from each recipient. Microcapsules were added to 500 μl acid-ethanol solution, sonicated and kept at −20°C. Results of insulin secretion are given in percentage of insulin content per islet. Insulin secretion activity and insulin content were compared with the preimplantation values.

Statistics

Values are presented as means ± SD (excepted when specified otherwise). The statistical significance of differences was tested by a one-way analysis of variance (ANOVA) with a Bonferroni posthoc test. The Mann-Whitney *U* test was used to analyze the insulin secretion of explanted capsules. The statistical tests were carried out using Systat version 8.0. Differences were considered to be significant at $P<0.05$.

RESULTS

Parameters Conditioning "In Vitro and In Vivo" Biocompatibility

Size of Microcapsules and $CaCl_2$ Concentration

After encapsulation, the mean diameter of empty capsules was 653±22 μm (n=460 from three independent experiments). Incubation of capsules for 18 or 24 hr in a culture medium supplemented with 0.3 mM $CaCl_2$ provoked a sig-

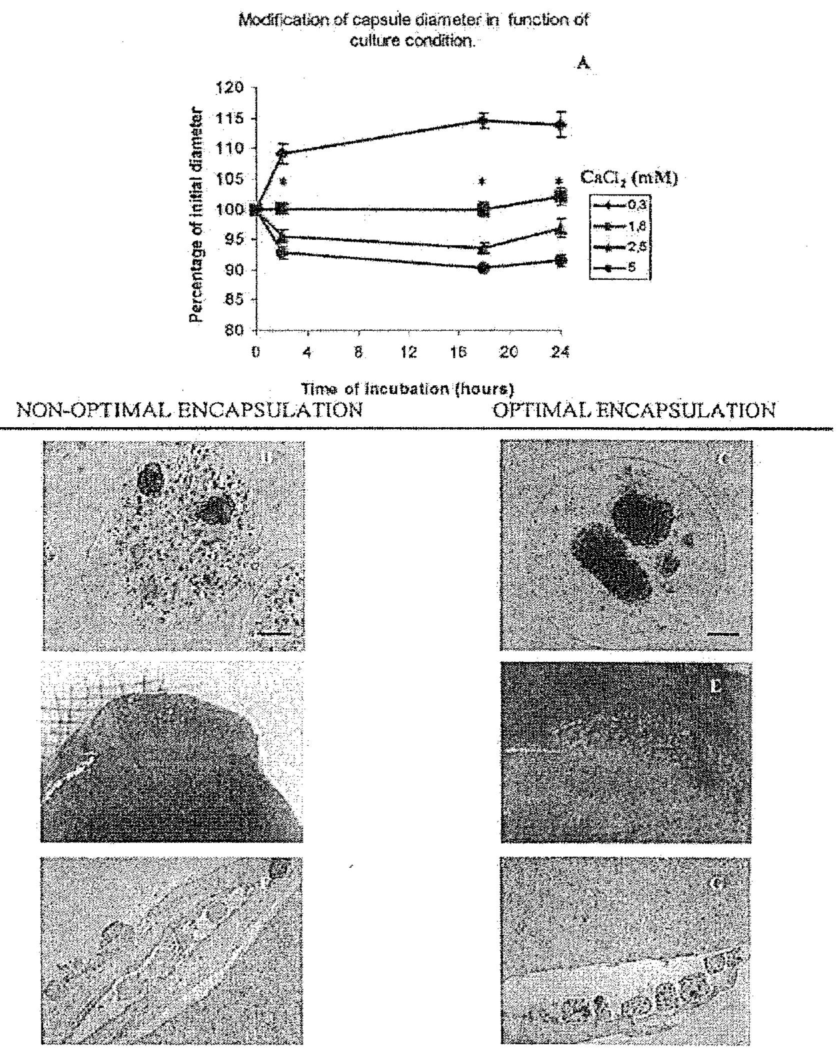

FIGURE 1. (A) Stabilization of the capsule diameter prior to transplantation. Stable capsule diameters were obtained in a medium containing 1.8 mM CaCl2 with an incubation time of 18 hr (*$P<0.005$ for capsule diameter with 1.8 mM vs. 0.3/2.5/5 mM $CaCl_2$). Significant swelling and retraction were observed in capsules incubated in medium containing low (0.3 mM) and high (2.5/5 mM) concentrations of $CaCl_2$ (300 capsules were evaluated for each series from three independent experiments), respectively. Transplantation of non-optimal (B) and optimal (C) encapsulated pig islets beneath the kidney capsule (location bar=100 μm). Note that graft fibrosis (D) and strong fibrosis (*) reaction, surrounding non-optimal capsules, were 1 month after implantation. No graft fibrosis and free-capsules were observed, 1 month after transplantation, in case of optimal capsules transplantation (E). In addition, insulin positive cells were found, at day 30 posttransplantation, inside optimal capsules (G; immunostaining for anti-insulin; original magnification: ×2.5) in contrast to capsules from non-optimal conditions (F; no staining for insulin; original magnification: ×2.5).

TABLE 1. Scoring of encapsulated pig islets after explantation

Animal number	Time of graft explantation	Macroscopic evaluation of graft (% of fibrosis)	Microscopic evaluation of graft (% of total capsule)			Porcine C-peptide (ng/ml) at day 30
			Degree of capsule overgrowth	Degree of broken capsule	Capsule containing DTZ^+ cells	
Non-optimal encapsulation						
FBS						
001	30	100	ND	ND	ND	0
002	30	100	ND	ND	ND	0
003	30	100	ND	ND	ND	0
Without preimplant 18-hr culture						
004	30	50	94	86	0	0.04
005	30	100	ND	ND	ND	0.03
Optimal encapsulation						
006	30	0	3	2	54	0.119
007	90	0	2	6	32	0.106
008	135	0	1	0	17	0.288
009	135	0	6	4	12	0.157
010	180	0	5	0	6	0.199
011	180	50	32	26	0	0.049
012	180	0	8	12	3	0.053

Capsules were removed, from the primate's kidney 30, 90, 135 and 180 days after transplantation. Two-independent investigators evaluated the grafts. The degree of cellular overgrowth, broken capsules and DTZ contained islets were microscopically evaluated on 400 capsules for each graft. ND, nondetermined since all the grafts were entrapped in fibrosis.

nificant swelling of capsules (+12.5% of initial diameter prior to culture (745±42 μm), $P<0.005$) (Fig. 1A). No significant modification of the diameter was observed for capsules incubated in culture medium supplemented with 1.8 mM $CaCl_2$ for 2 h (703±33 μm), 18 h (698±56 μm) or 24 hrs (707±69 μm). In contrast, incubation in medium with higher $CaCl_2$ concentrations (2.5 and 5.0 mM) induced a significant reduction in capsule diameter (−6.6%, $P<0.005$) as compared to the diameter obtained just after encapsulation.

Culture of Microencapsulated Islets Prior to Implantation

In order to stabilize the microcapsules, and obtain long term biocompatibility of encapsulated pig islets, preimplantation culture for 18 or 24 hr is necessary. Two primates that were immediately implanted after encapsulation demonstrated a strong cellular/fibrosis reaction against the graft after 1 month (Table 1, animals 004-005).

Three animals which received pig encapsulated islets cultivated prior to transplantation in CMRL 1066 supplemented with bovine serum, encapsulated islets were rapidly destroyed (<30 days) and massive cellular overgrowth and fibrosis were observed (animals 001-3) (Table 1; Fig. 1, D and F).

In these five primates, no porcine C-peptide was detected at day 30 posttransplantation (Table 1, Fig. 2). In contrast, primates transplanted with capsules cultured in optimal conditions demonstrated the biocompatibility of alginate encapsulated pig islets (see below, Table 1; Fig. 1, E and G).

Shape of Microcapsules

In order to obtain a long term biocompatibility of capsule, a regular, spherical shape of capsule is necessary (Table 1, Fig. 1C). One primate (animal 011) that received pig islets in capsules 38% of which were not well formed (Fig. 1B) displayed significant fibrosis (>50%) after 6 months. In this case, cellular overgrowth (32%) and capsular breakage (26%) were observed (Table 1). In the other primates that received a mean of 91±5% of well-formed capsules (Fig. 1C), fibrosis or cellular overgrowth was not evidenced.

A clear relationship between the capsule shape and the purity of islets preparations was evidenced. The exocrine contamination disturbs the laminar jet of the encapsulation device thereby increasing the proportion of non-well shaped capsules. In this study, the purity was constantly over or equal to 90% (94±2.6%: n=10) to be able to use our device.

Ideal Preparation of Alginate Microencapsulated Pig Islets

These preliminary data allowed us to determine the following best condition for in vivo experiments: the best regime was to culture pig encapsulated islets in 17 ml CMRL 1066 at 1.8 mM $CaCl_2$ for 18 hr, in 75 cm^2 non-tissue culture treated flask at a concentration of 10,000 capsules/ flask in a serum-free medium. In 6 out of 7 remaining primates, no graft fibrosis or cellular overgrowth was observed after 3, 4 or 6 months whether the microencapsulated pig islets were cultivated in the appropriate medium and when over 92% of capsules were well formed (Table 1).

Transplantation of Non-encapsulated Pig Islets and Empty Capsules

After transplantation of non-encapsulated pig islets under the kidney capsule of 2 primates, a peak of porcine C-peptide was evidenced 1 hr after transplantation (range 2.319−6.122 ng/ml). Porcine C-peptide was, however, below the detection threshold (<0.1 ng/ml) 7 days after transplantation (Fig. 2). After graft removal and tissue fixation, a strong

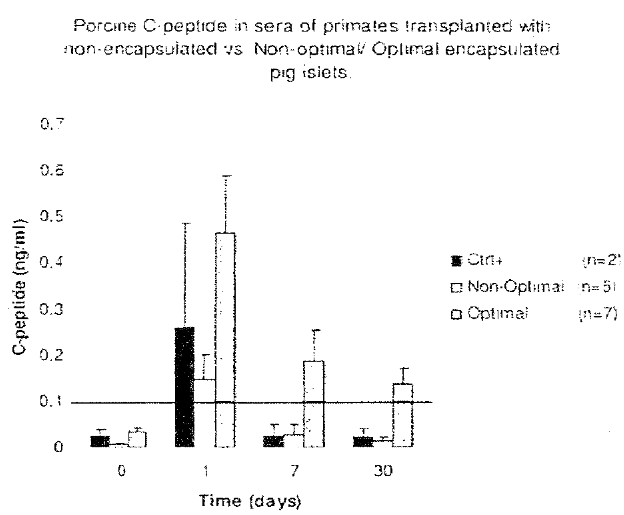

FIGURE 2. Porcine C-peptide level evolution in primates transplanted with non-encapsulated pig islets (n=2; Black) or non-optimal (n=5, White) / optimal (n=7; Grey) encapsulated pig islets. After 30 days of optimal capsules transplantation, porcine C-peptide was detected in 71% of transplanted animals (5/7 primates). No porcine C-peptide was detected 2 days after the implantation of free and non-optimal encapsulated pig islets (<0.1 ng/ml, black line). Mean ± SEM.

cellular reaction was observed 2 and 7 days after transplantation (Fig. 3). The cellular infiltration was essentially composed of CD3 (lymphocyte) and CD68 (monocyte-macrophage) positive cells (Fig. 3, B and C). Complement C3 deposition was evidenced on the graft, as soon as 2 days after transplantation (Fig. 3D). Empty capsules did not induced inflammatory and immunological reactions (data not shown).

Encapsulation Prolongs Pig Islet Survival

After stabilization, encapsulation of pig islets by high-M alginate improved graft survival after transplantation into non-diabetic primates (n=7). A mean level of 0.14±0.08 ng/ml of porcine C-peptide was detected until day 30 posttransplantation, in the sera of 7 primates. However, 2 primates had only a detectable level of porcine C-peptide (>0.1 ng/ml) until 7 days after transplantation (Fig. 2, Table 1). Level of C-peptide was significantly higher than the level obtained in naive animals (0.03±0.02 ng/ml). Dithizone and insulin positive cells were found in one graft after removal at day 30 posttransplantation (n=1). No fibrosis and no cellular overgrowth were observed in this graft (Table 1). Porcine C-peptide was detected in 2 recipients after 60 days of transplantation (0.266 and 0.193 ng/ml). Although no porcine C-peptide was detected in primate sera over 90 days posttransplantation, no graft fibrosis, no capsule overgrowth and insulin positive cells were observed (Table 1). After 135 (n=2) and 180 (n=3) days of transplantation, no graft fibrosis and fewer than 10% of capsules presenting cellular overgrowth were observed in 5 out of 6 primates (Table 1, Fig. 4, A and B). Dithizone positive cells were found inside grafts after 135 and 180 days of transplantation (Table 1, Fig. 4, C and D).

No significant modification of body weight and creatinine level was observed in the 7 primates receiving well encapsulated pig islets 4 to 6 months after implantation.

Microencapsulated Pig Islets Are Viable and Functional 4 and 6 Months after Implantation

Prior to transplantation, a mean viability of 84±6% (by Trypan Blue exclusion assay) was obtained for encapsulated pig islets (n=10). Transmission electron microscopy demonstrated the integrity of pig islets after encapsulation (data not shown). Prior to implantation, encapsulated pig islets (islets from 3 pig pancreases used for animals 008-12) demonstrated the capacity to produce insulin after glucose stimulation: an increased insulin release after exposure to glucose 15 mM supplemented with cAMP-raising agent (Forskolin 1 μM) was observed: 70.7±15.1% vs. 31.6±9.7% of insulin content for glucose 15 mM+Fsk 1 μM vs. glucose 5 mM, respectively. The mean stimulation index (SI), calculated as the fold-increase over baseline (% of insulin content at glucose 5 mM), was calculated at 2.5±1.1.

Capsules were removed 135 (n=2) and 180 (n=3) days after transplantation and were incubated in the presence of different concentrations of glucose to assess the function of pig islets from explanted capsules. An increase in insulin release, after exposure to glucose 15 mM supplemented with Forskolin, was observed for pig encapsulated islets removed at day 135 (animals 008-009): 6.6±2.3% vs. 2.9±0.9% of insulin content for glucose 15 mM+Fsk 1 μM vs. glucose 5 mM ($P=0.028$, n=2; Fig. 4E). The mean SI was calculated at 2.2 (range 2.0–2.7). After 180 days of transplantation, one graft did not demonstrate insulin content and the capacity to secrete insulin. This correlated with graft fibrosis and capsule overgrowth (Table 1, animal 011). Among the 2 other cases (animals 010-012), one pig islet graft demonstrated the capacity to increase insulin secretion after exposure to glucose 15 mM+Fsk 1 μM (8.4±3.7% vs. 4.7±1.9% of insulin content for glucose 15 mM+Fsk 1 μM vs. glucose 5 mM) corresponding to a SI at 1.8 (Fig. 4F). Follow up at the same time, observed insulin secretion, but no response to increasing glucose concentrations or the cAMP-raising agent were demonstrated in the second animal.

However, a significant decrease in insulin content was observed in capsules explanted from primates after 135 (2.2±1.9 ng/islet) and 180 (1.1±1.0 ng/islet) days of transplantation ($P<0.005$) as compared to those extracted from capsules prior to transplantation (32.2±24.3 ng/islet) for capsules containing a mean of 2–3 pig islet cells, $P<0.005$).

Transplantation of Encapsulated Pig Islets Elicits a Humoral Immune Response

Sera from 12 animals transplanted with encapsulated pig islets were analyzed, by Flow Cytometry, and compared to animals receiving non-encapsulated pig islets (n=2) and empty capsules (n=1). In all primates, the presence of anti-pig antibodies (IgM and IgG) was detected prior to transplantation thereby confirming the presence of preformed anti-pig antibodies (Fig. 5). No increase in IgM or IgG anti-pig antibodies was found in the sera of primates transplanted with empty capsules. In contrast, when primates were given non-

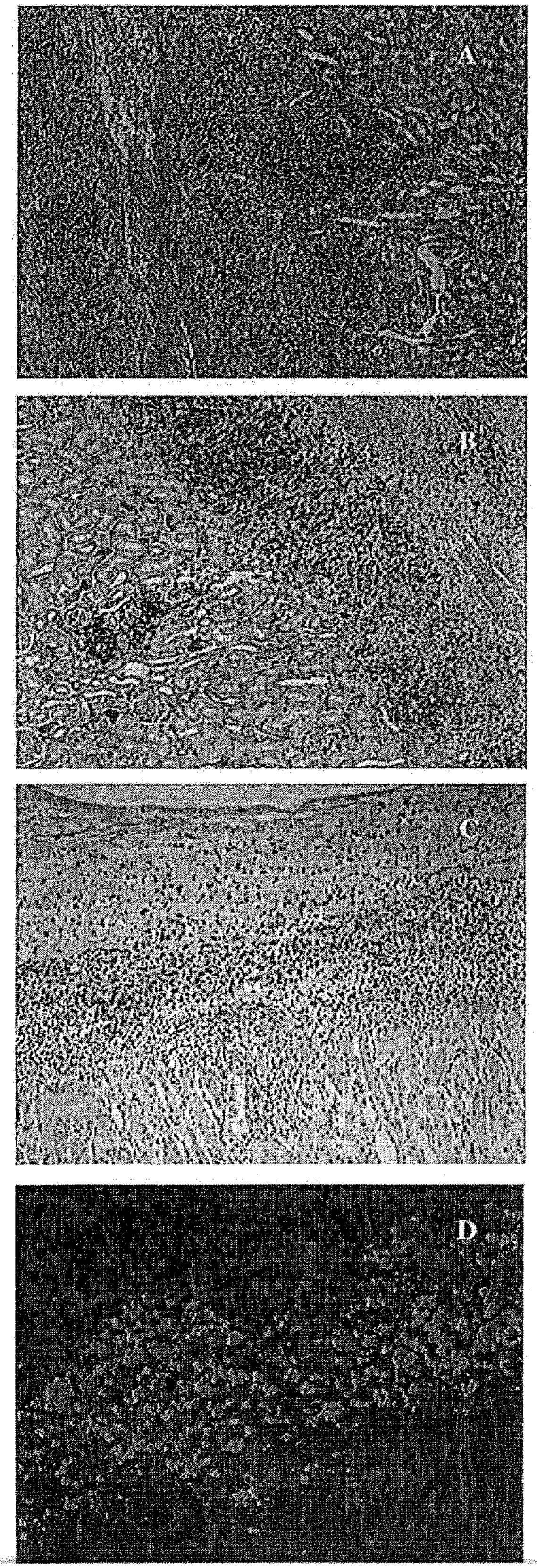

FIGURE 3. Cellular infiltration and graft destruction were observed 7 days after non-encapsulated pig islet transplantation. Very few insulin-positive cells were found ((A) insulin staining; original magnification: ×5). Immunohistochemistry revealed massive CD3 (B) and CD68 infiltration (C) (original magnification: ×5). Complement C3 deposition was observed on the graft (original magnification: ×10, D).

encapsulated pig islets (n=2) the level of anti-pig IgM and IgG antibodies was strongly increased. When badly encapsulated islets (n=2), islets cultivated in bovine serum (n=3) or well encapsulated pig islets (n=7) were implanted a significant increase in the level of anti-pig IgM and IgG antibodies was observed as shown in Figure 5.

DISCUSSION

The first aim of this study was to demonstrate the biocompatibility of encapsulated pig islets for long-term (6 months) in primates and to evidence the parameters which significantly influence the compatibility of these capsules. The second major goal was to proof the concept by demonstrating that pig encapsulated islets are capable to respond to a hyperglycemic stimulus 6 month after implantation into the most stringent xenogeneic model and without immunosuppression.

Without encapsulation, pig islets are destroyed within 2 days of transplantation as evidenced by loss of C-peptide production and humoral and cellular rejection at immunohistology (*16*).

In order to prolong porcine islets viability without immunosuppression, pig islets were encapsulated following several parameters which allowed long-term viability and immunoprotection (*17, 18*). Pig islets were encapsulated in a highly purified high-M alginate cross-linked with Ca^{++} ions and the use of a very low endotoxin-containing alginate without additional permselective PLL coating seemed crucial (*9, 19*). Similarly, Duviver-Kali et al. and Omer et al. reported improved capsule integrity and pig islet viability up 3 months after transplantation in B6AF1 mice, by using similar alginate (*10, 20*).

Although recent experiments demonstrated improved stability after alginate cross-linkage with Ba^{++}, we tested alginate capsule cross-linkage with Ca^{++} followed by a preimplantation culture at a $[Ca^{++}]$ concentration of 1.8 mM (*4, 5*). This $[Ca^{++}]$ concentration was chosen after testing several lower and higher concentrations of $CaCl_2$ during preimplantation culture up to 24 hr. Although Ham F10/F12 medium corresponds to the optimal culture media for non-encapsulated adult pig islets culture, low $[CaCl_2]$ (0.3 mM) media induce capsule swelling and provoke disruption of capsule membranes which could expose pig tissue to the host immune system and thereafter to graft fibrosis and islet necrosis (*17, 21*). Following these in vitro data, the optimal concentration of 1.8 mM was selected as well as a culture period of 18 hr prior to implantation in order to stabilize the alginate capsules. Several commercial media contain a concentration of 1.8 mM $CaCl_2$ and CMRL 1066 was chosen to culture encapsulated pig islets since its efficacy for pig islet cultures is known (*22*). In three primates, encapsulated pig islets were

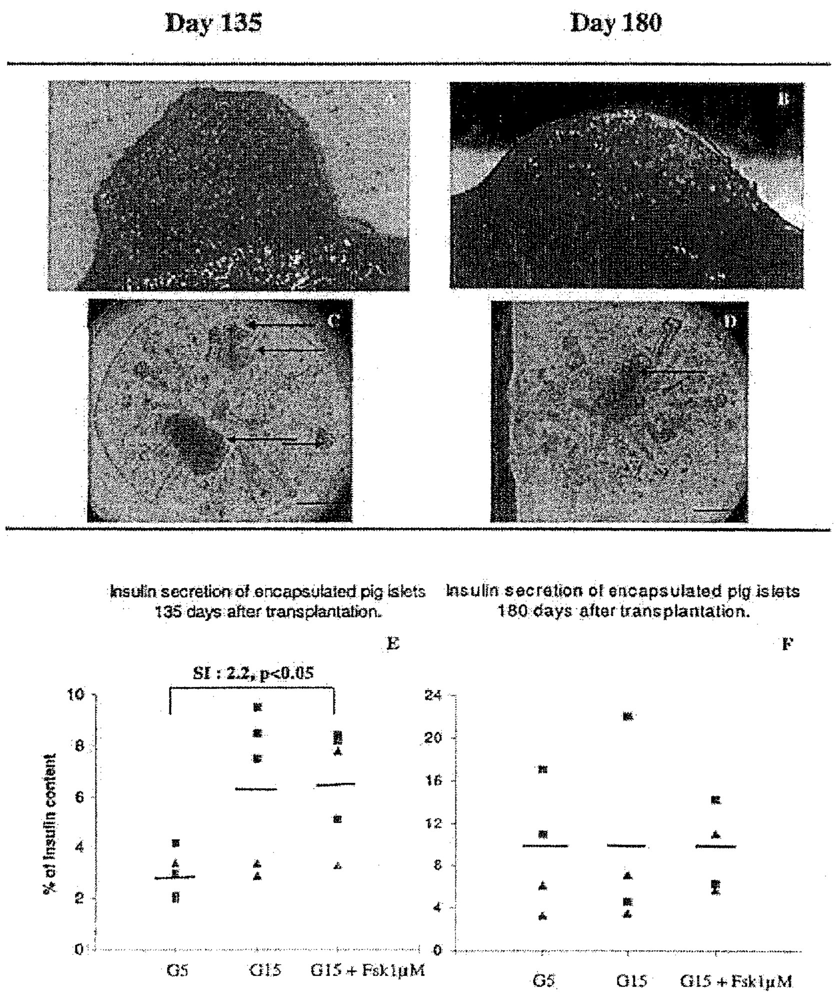

FIGURE 4. No fibrosis was observed macroscopically for grafts removed after 135 and 180 days of transplantation in primates (A, B, *graft). Dithizone positive cells (arrow) were also detected for pig encapsulated islets explanted after 135 (C) and 180 (D) days (location bar: 100 μm). Encapsulated pig islets explanted from grafts 135 days after transplantation were able to produce a significant amount of insulin after glucose/Forskolin stimulation (E), ($P<0.05$ for G15+Fsk vs. G5, n=2). After 180 days, insulin secretion was observed but without response to stimulation (F, n=2). Black square and black triangle represents replicates for each primate.

cultured in CMRL medium complemented with bovine serum, and severe graft destruction ensued one month after implantation. Although capsules were intensively washed prior to transplantation, bovine proteins could stick to the capsule surface and lead to the host immune system activation. Therefore, serum-free CMRL was then used to culture encapsulated pig islets in the following seven in vivo implantations and graft destruction was not evidenced.

The percentage of well-formed capsules also appears crucial for pig encapsulated islet biocompatibility. In fact, one primate which received stabilized encapsulated pig islets cultivated in free-serum medium, demonstrated 50% graft fibrosis 6 months after implantation. Retrospectively, this graft was composed by a high percentage (38%) of pig islets protruding outside the capsules (*23*).

Overall, these data suggest that encapsulated pig islets must be embedded in very pure alginate, cultivated for 18 or 24 hr in serum-free medium containing a concentration of 1.8 mM of $CaCl_2$. In addition, the ratio of well formed capsules must be over 90% to obtain a long term in vivo biocompatibility in the pig to primate model.

Although the survival of encapsulated pig islets in diabetic monkeys was reported nine years ago but never confirmed by others teams (*7*), there is one recent and casuistic manuscript describing biocompatibility of alginate/polyornithine/alginate microcapsules after 8 weeks of implantation into non-diabetic primate (*24*). The present experimental work in vivo clearly demonstrates that implantation of optimized capsules might improved pig islet survival into primates without immunosuppression for up to 6 months. In this study, the monkeys were not diabetic since the first goal was to assess the biocompatibility of pig encapsulated islets and to prove the concept. The more important result obtained in this study was certainly the demonstration that 135 or 180 days after the implantation of encapsulated pig islets, some of the islets survived and were able to respond in vitro to a glucose challenge showing a stimulation index up to 2.2. This result demonstrates that pig islets survived up to 6 months in the most stringent xenogeneic pig to primate model without any immunosuppression. Evidently, among the explanted capsules there was a strong reduction of insulin content as compared to pretransplantation level (from 30 ng/islet up to 2 ng/islet), but the loss of surviving beta cells probably derived from multiple origins: the fact that (i) capsules

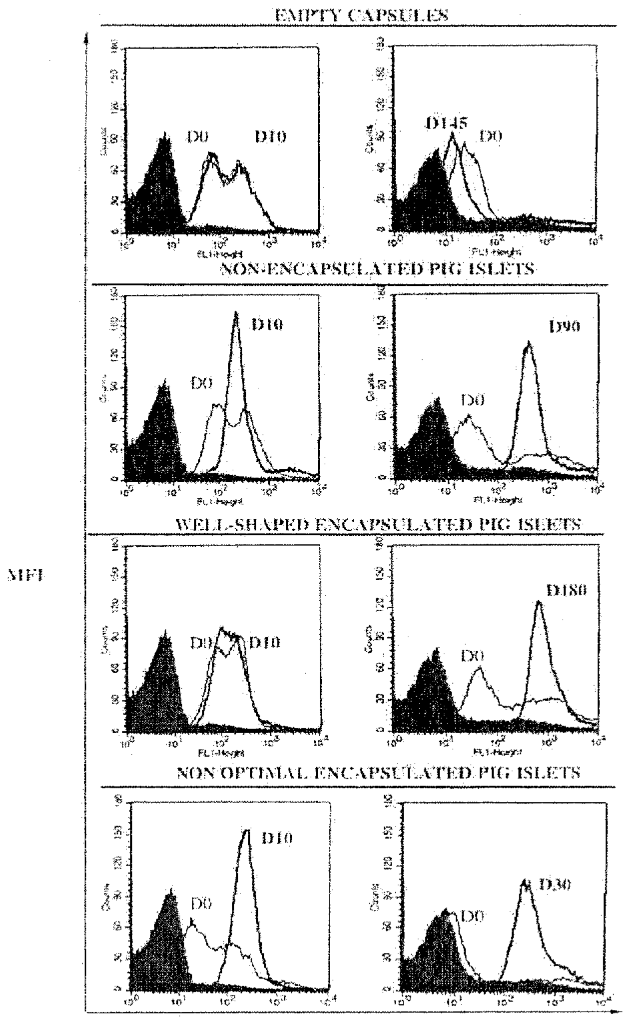

FIGURE 5. Representative development of anti-pig antibodies in primate sera prior to and after transplantation with empty capsules, non-encapsulated pig islets, well-shaped encapsulated pig islets (stabilized) and non-optimally encapsulated pig islets (left: IgM, right: IgG). All animals had preformed antibodies. When empty capsules were implanted, no change in the anti-pig IgM or IgG was observed, while in all animals receiving encapsulated pig islets a significant increase in IgG was evidenced thereby confirming immunisation.

were implanted in normoglycemic monkeys, that (ii) capsules were not injected following a monolayer which certainly induced a lack of oxygenation on the external layers of cells, that (iii) chronic rejection ensues due to continuous anti-pig IgG production or eventually, that (iv) cytokine release by encapsulated islets occurred (*18*). Regarding this latter parameter, however, it must be pointed out that cytokine release was clearly identified as a mechanism of islets loss when encapsulated islets were injected into the peritoneum which seems a more inflammatory site than kidney space (Dufrane et al., manuscript submitted).

The site implantation under the kidney capsule was, used in this work, since local oxygen pressure is high and the recovery of capsules is rendered easy for analysis, but for clinical applications other sites should be investigated i.e. as subcutaneous space. The usual intra-portal way, used in clinical applications, would be impossible due to the capsules size which will induce hepatic thrombosis.

Some of the pig islets survived long-term despite a strong humoral anti-pig immune response. In fact, all the primates used in this study had preformed anti-pig antibodies of both IgM and IgG types. Despite the encapsulation, all primates developed an elicited anti-pig immune response as evidenced by the significant shift of both anti-pig IgM and mainly IgG antibodies by flow cytometry. When empty capsules were implanted under the kidney capsules, no humoral response was elicited up to day 145 after grafting thereby demonstrating that pig islets were responsible for the antibody response. Despite this antibody production, no rejection or fibrosis was evidenced thereby demonstrating the immune protection of the pig islets by the capsules (*10*). The immunization against pig proteins could be the consequence of a small percentage of pig islets not being encapsulated or simply prove that pig proteins might get out of the capsules (*25*), such as porcine C peptide (*26*).

In conclusion, this study demonstrated that several parameters must be in place to improve the biocompatibility and survival of encapsulated pig islets up to 6 months in the most stringent xenogeneic pig to primate model. This partial but long-term survival is obtained despite an ongoing anti-pig IgG response thereby showing that encapsulation protects the islets in the long term. Similar experiments are being performed in STZ-induced diabetic monkeys to demonstrate the usefulness of this approach in type I diabetes and the possible correction of hyperglycemia in vivo. In addition, the cellular graft is now being designed as a mono-layer graft to improve the oxygenation of beta cells.

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